

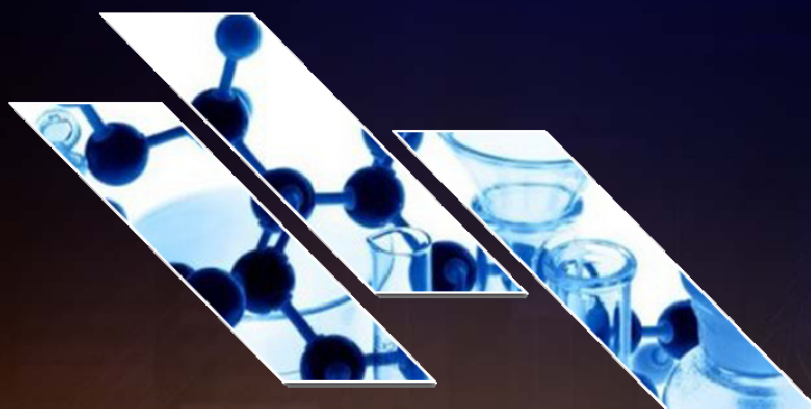


# EDGEWOOD CHEMICAL BIOLOGICAL CENTER

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## NOVEL CELL-BASED ASSAYS FOR DETECTING LOW LEVELS OF ACTIVE RICIN FOLLOWING DECONTAMINATION



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## PREFACE

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## CONTENTS

1.	INTRODUCTION .....	7
1.1	Ricin Toxin .....	7
1.2	Ricin Detection .....	9
1.2.1	Antibody-Based Detection.....	9
1.2.2	Cytotoxic MTT Viability Assay .....	9
1.2.3	Inhibition of <i>In Vitro</i> Protein Synthesis.....	10
1.2.4	Assay with Engineered Cell Line .....	10
1.3	Project Goals and Specific Objectives.....	10
2.	MATERIALS AND METHODS.....	11
2.1	Cell Line and Plasmid Specifications .....	11
2.2	Plasmid DNA Isolation.....	12
2.3	Transfection and Selection of Recombinant Cell Line.....	12
2.4	Routine Maintenance of Cell Line.....	13
2.5	Assay Protocol and Optimization Parameters.....	13
2.6	Determination of Cell Line Sensitivity .....	14
2.7	Primary Data Acquisition and Analysis.....	14
2.8	Coupon Inoculation and Sample Preparation .....	15
2.9	Decontamination Assay .....	15
2.10	Ricin Limit of Detection Estimations .....	15
3.	RESULTS .....	16
3.1	Cell Line Clone Selection .....	16
3.2	Assay Optimization.....	17
3.2.1	Cell Concentration and Incubation Time.....	17
3.2.2	Sensitivity of Cell Line to Pure Ricin Standards .....	18
3.2.3	Decontamination Sample Preparation.....	22
3.3	Ricin Decontamination with Decontamination Solutions.....	22
4.	DISCUSSION AND CONCLUSIONS .....	23
	LITERATURE CITED.....	25
	APPENDIX: GENERAL RICIN BIOASSAY PROTOCOL.....	27

## FIGURES

1.	Mode of Action for Cytotoxicity of Ricin Toxin.....	8
2.	Effect of Cell Number and Incubation Period on Luciferase Expression.....	18
3.	Sensitivity of Two Cell Lines to Varying Amounts of Holoricin.....	19
4.	Effect of Low Levels of Holoricin on Luciferase Expression .....	19
5.	Optimization of Ricin Addition .....	20
6.	Response Reproducibility of Clone 1-2 Cells to Ricin.....	21
7.	Linear Relationship between Clone 1-2 and Ricin Concentration.....	21

## TABLES

1.	List of Cell Lines and Plasmids .....	11
2.	Clonal Selection Results .....	16
3.	Effect of Neutralized Disinfectant on Luciferase Induction .....	22
4.	Decontamination of Crude Ricin on Steel Coupons with Bleach, Chlorine Dioxide, and Hydrogen Peroxide.....	23



# NOVEL CELL-BASED ASSAY FOR DETECTING LOW LEVELS OF ACTIVE RICIN FOLLOWING DECONTAMINATION

## 1. INTRODUCTION

Ricin is a potent protein toxin derived from the seed of the castor bean plant, *Ricinus communis*. In laboratory mice, the LD<sub>50</sub> (the dose of a chemical that kills 50% of a sample population) is largely dependent on the mode of exposure, with inhalation being the most lethal. It is a heterodimeric ribosome-inactivating protein with A and B chains. Each chain serves a distinct function. The B chain is required for binding to the mammalian cell surface and the A chain enzymatically cleaves an adenine nucleotide from 28S ribosomal ribonucleic acid (RNA), resulting in the inhibition of protein synthesis, and subsequently, cell death. Most ricin detection assays are based on only the detection of the A chain or simply the presence of protein, and as such, have significant limitations with respect to the detection of functional holoricin protein.

Through a collaborative interagency agreement funded by the U.S. Environmental Protection Agency (EPA) National Homeland Security Research Center (NHSRC), the U.S. Army Edgewood Chemical Biological Center (ECBC) and NHSRC have developed a novel bioassay for detecting functional holoricin following decontamination procedures.

The specific objectives for this project are listed below:

- Develop a novel cell-based assay for functional ricin.
- Investigate the use and application of the newly-developed bioassay in confirming the absence or presence of functional toxins in post-decontaminated samples from building interior surfaces.

This report summarizes the work performed and the successful completion of these objectives. The resulting optimized cell-based assay can reliably detect very low levels of holoricin. In addition, this assay is easily adaptable to a high-throughput format.

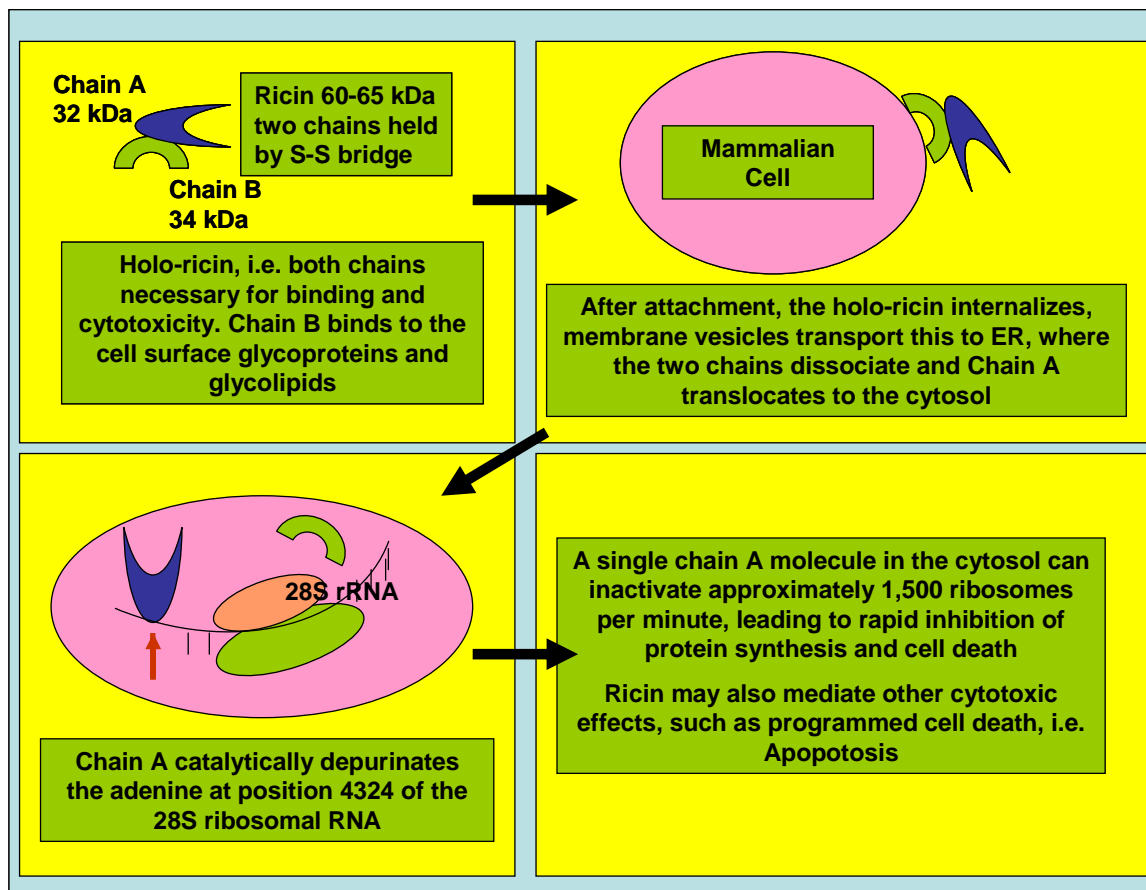
### 1.1 Ricin Toxin

The ricin protein toxin makes up 1–5% by seed weight of the castor bean plant, *R. communis*. The *R. communis* plant is cultivated in southern U.S. It also grows as a weed. The whole plant is poisonous, containing the toxin ricin with the seeds having the highest concentration of poison. Each fruit contains three mottled seeds, 5–15 mm long. One to three seeds may be fatal to a child. In an adult, two to four seeds may cause poisoning while eight may be fatal.

The LD<sub>50</sub> and time to death levels for ricin in laboratory mice (Stirpe, 1986, 2004; Barbieri, 2004; Griffith, 1994) are given below:

- 3–5 µg/kg and 60 h by inhalation
- 5 mg/kg and 90 h by intravenous injection
- 20 mg/kg and 85 h via intra-gastric ingestion

As a heterodimeric ribosome-inactivating protein, ricin (66 kDa in size) is made up of A (267 amino acids in length, 32 kDa) and B (267 amino acids in length, 34 kDa) chains, which are linked via disulfide bonds. Both chains are glycoproteins and must be linked for the holo-ricin to be toxic to mammalian cells. The B chain is lectin specific for binding two galactose sugars and can bind to the mammalian cell surface. The mode of ricin cytotoxic action is summarized in Figure 1. Upon binding to the cell surface, the ricin toxin is internalized. The ricin A chain enzymatically cleaves 28S ribosomal RNA at adenine nucleotide (A4324) near the 3' end of the polynucleotide chain (Barbieri, 2004; Lord, 1994; Heisler, 2002). This deletion results in the failure of elongation factor-2 to bind to the ribosome and, thereby, inhibits protein synthesis, which results in cell death (Figure 1). The ricin A chain has a  $K_m$  (binding affinity) of 0.1 µM and an enzymatic constant of 1,500/min for the cleavage of A4324 (Franz, 1997).



**Figure 1. Mode of action for cytotoxicity of ricin toxin**

## 1.2 Ricin Detection

Currently, detection of ricin toxin is based on one of the following approaches:

- a) Detection of surface antigen on the protein toxin
- b) General loss in mammalian cell viability due to inhibition of protein synthesis
- c) In vitro protein synthesis inhibition of the reporter gene, luciferase
- d) Cell-based assay, engineered with a reporter gene

### 1.2.1 Antibody-Based Detection

Historically, one of the most common approaches for detecting protein toxins is based on detection of antigens using antibodies. This is done using electrochemiluminescence (ECL) based technology such as the MIM Analyzer instrument and kits, which are commercially available and marketed by BioVeris/Roche (Gaithersburg, MD). ECL uses a sandwich immunoassay format in which anti-ricin antibodies (capture antibodies) are biotinylated and pre-bound to Streptavidin-coated paramagnetic beads. Detection of the ricin antigen is facilitated by the use of a second anti-ricin antibody labeled with ruthenium trisbipyridine chelate (RU) as a reporter antibody. Presence of the target antigen links the reporter antibody to the capture antibody on the paramagnetic beads. The ECL analyzer collects the beads on the surface of an electrode using a magnet and rids the sample of unbound non-specific material with a washing step. The complexed ricin antigen labeled with RU and tripolamine present in the reaction buffer are activated by oxidation at the electrode. The tripolamine loses a proton and becomes a powerful reducer for the RU, resulting in excited-state RU. The excited-state RU then returns to its ground-state after emission of a photon at 620 nm, which is detected by the analyzer. Even though the ECL detection limit is low ( $\leq 1$  ng/mL), antibody-based detection provides no information with regards to whether the ricin is biologically active.

### 1.2.2 Cytotoxic MTT Viability Assay

Mammalian cell line viability and proliferation can be readily assayed by the use of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], which is converted to a purple formazan dye by cellular reductive power including the pyridine nucleotide cofactors, NADH/NADPH. Production of the reductive cofactors and MTT reduction can only be carried out in metabolically active cells. Cessation of protein synthesis, caused by catalytic cleavage of ribosomal RNA in the presence of functional ricin, leads to a general decay in cell viability. Loss in cell viability is detected as loss in absorbance of formazan at 550–620 nm, resulting from the reduction of MTT by NADH/NADPH in the cell. Because generation of NADH/NADPH is dependent on a functional respiratory pathway and intact mitochondria, any shock or treatment impacting this metabolic machinery is expected to give false-positive results even in the absence of functional ricin.

### 1.2.3 Inhibition of In Vitro Protein Synthesis

Because the ricin A chain catalytically depurinates A4324 from 28S ribosomal RNA, a rapid loss of protein synthesis is the mode of action for active toxin. Luciferase gene expression in nuclease treated cell-free rabbit reticulocyte lysate and monitoring of its activity provide a rapid approach for determining the presence of active ricin toxin. A microtiter plate-based assay has been developed (Hale, 2001) to monitor the presence of active ricin toxin in a sample. Inhibition of luciferase activity was observed in the presence of the single A chain and holoricin. A semi-linear relationship between luciferase activity was reported to be 0.001–0.15 nM and 0.15–0.6 nM (1 nM = 66 µg/mL), for A chain and holoricin, respectively. As the ricin B chain is responsible for the initial binding, the entry, and the internalization of holoricin within the cells, detection based on in vitro protein synthesis is relevant to the catalytic A chain activity only. The presence of active or functional holoricin cannot be ascertained by such an approach.

### 1.2.4 Assay with Engineered Cell Line

Reporter genes, such as the luciferase enzyme or green fluorescent protein, are powerful tools, which have been used for detection assays for ricin protein (DeWet, 1987). Zhao et al. (2005) have reported a highly sensitive luciferase-based assay for bacterial toxins; however, this assay requires viral transduction before each assay, which increases the time necessary to perform the assay and introduces assay-to-assay variability. Recently, Halter et al. (2009) reported development of a mechanistic bioassay by engineering a Vero cell line with a reporter gene, green fluorescent protein (GFP). A stable transfectant Vero cell line expressed the reporter gene in a constitutive manner and responded to the presence of ricin within 6–8 h of contact. Quantitative microscopy in conjunction with flow cytometry was used to measure ricin response. This is a functional assay, but it requires sophisticated microscopy and specially trained personnel. Furthermore, such an assay is not scalable and has not been adapted for post-decontamination analysis of residual ricin.

## 1.3 Project Goals and Specific Objectives

The purpose of this project was to advance the current science of functional ricin detection based on the binding of the B chain to the cell surface and subsequent cessation of protein synthesis via catalytic depurination of the 28S ribosomal subunit by the functional ricin A chain. The overall goal included providing guidance on the selection of decontamination technologies by confirming the presence of functional ricin in environmental samples. The specific objectives have been listed in Section 1.

The strategy for developing the cell assay included the use of an engineered human-derived cell line harboring a reporter gene that yields a quantifiable and dosimetric response in the presence of functional ricin. For this effort, a luciferase gene was stably transfected into the cell line, and the resulting cell clone was used to develop an optimized luciferase assay for the detection of functional ricin. To avoid the cytotoxic effects of constitutive luciferase expression, the reporter gene was placed under the control of a repressible promoter (Tet-Off). Ultimately, a cell line was constructed that could be used for the detection of low levels of functional ricin.

## 2. MATERIALS AND METHODS

Development of a bioassay required the setting up of a dedicated mammalian culture laboratory. The cell-lines, plasmid containing the luciferase gene TRE promoter, and the culture media were obtained from commercial sources. A description of the cell lines and genetic markers used to construct the double stable cell line are listed in Table 1. Table 2 lists suppliers and catalog numbers for the materials used in this project.

### 2.1 Cell Line and Plasmid Specifications

**Table 1. List of Cell Lines and Plasmids**

Cell Line or Plasmid	Source	Description
HeLa Tet-Off Advanced Cell Line	Clontech	Cell line used as recipient, produces Tet repressor and has a G418 resistance marker
pTRE-Tight-Luc Plasmid + Linear Hygromycin Marker	Clontech	Recombinant plasmid containing luciferase gene under Tet responsive element used as donor DNA for double transfectant
<b>Double Transfectant</b>	<b>This project</b>	<b>Stable double transfectant resulting from introduction of pTRE-Tight-Luc plasmid into HeLa Tet-Off cells</b>
CHO-AA8-Luc Cell Line	Clontech	Positive control cell line, derived from Chinese hamster ovary, stably transfected with luciferase gene under control of Tet-Off system

The HeLa cells used to construct the doubly-stable cell line were derived from a human cervical epitheloid carcinoma and are commonly used for bioassays. The HeLa Tet-Off cells used for this project have been transfected with the tetracycline-controlled transactivator gene, producing a TetR-fusion repressor protein. This cell line has been well characterized by the commercial supplier, Clontech (Mountain View, CA). Clontech has determined the doubling time for these cells to be approximately 20 h during log phase. The cell morphology is reported to be adherent and elongated with 2 or 3 filopodia. The cells were maintained in complete media containing 90% Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% Tet System approved Fetal Bovine Serum (FBS), 4 mM L-glutamine, 100 µg/mL G418, 100 units/mL penicillin G sodium and 100 µg/mL streptomycin sulfate. Trypsin Ethylenediaminetetraacetic acid (EDTA) and phosphate buffered saline (PBS) were used for routine cell passage.

The positive control cell line used for this project was CHO-AA8-Luc. It was maintained in 90% Eagle Minimum Essential Medium supplemented with 10% FBS, 4 mM L-glutamine, 100 µg/mL G418, 100 units/mL penicillin G sodium, 100 µg/mL streptomycin sulfate, and 100 µg/mL hygromycin B. This cell line is very similar to the stable double transfectant developed in this project. However, it was derived from a Chinese hamster ovarian (CHO) cell line. Although it expresses high levels of luciferase upon induction, it is not a human derived cell line. Consequently, sensitivity of this cell line to ricin will likely differ from that of the HeLa cell

line. Data presented in the Results section of this report suggest that these cells are less sensitive to ricin toxicity.

## 2.2 Plasmid DNA Isolation

To propagate the plasmid used for transfection, pTRE-Tight-Luc, chemically competent *Escherichia coli* DH5 $\alpha$  cells were used for heat-shock transformation of the plasmid and subsequent growth. The plasmid was purified using a Qiagen midi-prep kit. The plasmid prep was quantified and a quality assessment was conducted using spectrophotometric readings at wavelengths of 260 and 280 nm. Further confirmation was performed on the plasmid prep by restriction mapping. The clone of interest was subjected to an enzymatic double digestion with BamHI/Nhe I and a single digestion of Xba I. The expected sizes of digested fragments were verified following separation by electrophoresis on a 2% agarose/tris-acetic acid-EDTA gel and visualization under UV light following staining with ethidium bromide (data not shown).

## 2.3 Transfection and Selection of Recombinant Cell Line

A transfection procedure was used to develop a doubly-stable Tet-Off cell line expressing the luciferase gene. The liposomal reagent Clonfectin was used to transfect the pTRE-Tight-Luc vector into the HeLa Tet-Off cells. This procedure results in stable transfectants when plasmid DNA randomly inserts into the host chromosome. Luciferase expression is dependent on the number of copies inserted and the insertion location. To obtain stable transfectants, pTRE-Luc plasmid DNA was cotransfected with a hygromycin linear selection marker. Clones that grew on media containing hygromycin were then screened for luciferase expression to determine if the luciferase gene had been stably inserted as well. This strategy required the screening of many hygromycin-resistant colonies, as some were not transfected with both genetic elements, the selection marker and the plasmid.

For the transfection procedure, HeLa cells were seeded in a 6-well plate at a density of approximately  $2 \times 10^5$  cells/well. The cells were grown for 18–24 h to 60–90% confluency. The pTRE-Tight-Luc plasmid was then cotransfected with the linear hygromycin marker. The Clonfectin reagent was first added to the plasmid DNA and then to the cells following the vendor's protocol. After 4 h incubation, the transfection cocktail was removed, the cells were washed, and fresh complete media was added. The transfected cells were incubated at 37 °C for 24 h prior to hygromycin selection. Complete media containing 200  $\mu$ g/mL hygromycin B was used for the selection of clones successfully transfected with both plasmids. Hygromycin-resistant clones were analyzed microscopically and locations of viable clone clusters were marked on the dish. The cells were removed using cloning cylinders. The media was removed from the dish, the cloning cylinder was placed on the clone, and trypsin was added to the cloning cylinder to remove the cells. The cells were pipetted out of the cylinder and placed in a 24-well dish with fresh media. Isolated clones were moved to larger growth vessels as necessary.

All clones that survived the selection process and grew to sufficient numbers were grown in the absence or presence of 100 ng/mL doxycycline, resulting in induction or repression of luciferase, respectively. Either doxycycline or tetracycline can be used for the suppression of luciferase induction, as the system responds equally well to both. However, doxycycline was

used instead of tetracycline for maintenance of a stable cell line because it has a much longer half-life. Induction of luciferase activity was measured using a standard luciferase assay. Initially, luciferase was assayed following incubation for up to 72 h in the presence or absence of doxycycline. Induction of protein expression was assayed by adding luciferase assay reagent to the cells, which results in complete lysis of the cells and generation of a luminescent signal. The signal was measured using a luminometer. Clones expressing the highest levels of luciferase upon induction, and the lowest level of background expression under uninduced conditions (resulting in a high signal to noise ratio), were selected and further passaged. All selected clones were expanded to multiple flasks. Two flasks were used to make frozen cell stocks. The cells were frozen in freezing media using the vendor recommended protocol.

## 2.4 Routine Maintenance of Cell Line

Clonal cell lines were split in the presence of doxycycline twice a week to maintain the desired cell confluency needed for healthy cell growth. The typical split ratio (the cell culture dilution) was 1:5, but varied depending on the size of the culture vessel and age of the culture. Cells were washed with sterile Dulbecco's phosphate buffer saline solution (D-PBS) before they were detached from the flask surface using trypsin-EDTA solution. Once the cells detached from the vessel surface, the trypsin-EDTA was neutralized with complete DMEM. All but 20% of the suspended cell volume was discarded, and fresh complete DMEM media containing doxycycline was added to the remaining cells.

For inducing the luciferase gene expression, the cells were trypsinized from the flask and collected by centrifugation at 100 x g for 5 min in a 50 mL conical tube. The supernatant was removed and the cells were washed by resuspension in D-PBS and then collected again by centrifugation. This wash procedure was repeated three times in complete DMEM media lacking doxycycline. The cell titer was estimated by mixing an aliquot of cells with trypan blue in a 1:10 ratio. Cells were enumerated in a 10  $\mu$ L aliquot using a hemacytometer. Based on the cell count, the cells were then diluted to a concentration of  $10^5$  cells/mL using fresh complete DMEM without doxycycline, and a 100  $\mu$ L aliquot of cell suspension was seeded in each well of a micro titer plate.

## 2.5 Assay Protocol and Optimization Parameters

The general protocol for the luciferase assay was as follows (flow chart in the Appendix):

- Cells were seeded in a 100  $\mu$ L/well volume in a 96-well micro titer plate at an optimal density ( $10^4$ – $10^5$ /well), which was determined during the assay optimization, and based on incubation requirements and expression levels.
- Ricin toxin samples or decon samples in a 25  $\mu$ L/well volume were added to the cells and incubated for a predetermined amount of time. The time of ricin addition after cell seeding was determined during the assay optimization. It was based on the time required for cells to adhere to the plate surface, the time required for toxin entry into the cell, and the subsequent detection by inhibition in luciferase expression. In addition, preliminary experiments were conducted to determine the

dilution extent of the necessary post-decon samples for eliminating the toxic effects of the decontamination by-product(s) or residual decontamination agents.

- Cells were lysed by adding an equal volume (125  $\mu$ L) of Promega (Madison, WI) One-Glo reagent.
- Light output was then measured within 5 min on a Promega GloMax luminometer.
- Typically, each sample was taken through eight-point 1:2 dilutions: 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, and 1/256.

As stated, Steps 1 and 2 required optimization prior to the analysis of the decontaminated samples. Controls used for assay development and optimization included induced (– dox) and uninduced (+ dox) cells. Induced cell controls were included to determine the highest signal levels resulting from luciferase expression. Uninduced cell controls were used to determine background signal levels. These control values were used for data analysis.

To determine the optimal cell concentration per well and appropriate induction time, two cell concentrations were tested in a time course experiment. Wells of a 96-well plate were seeded with either  $10^4$  or  $10^5$  total cells per well. Three assay plates were set up to assess luciferase induction levels at three distinct time points, 24, 48, or 72 h.

## 2.6 Determination of Cell Line Sensitivity

Following the determination of cell concentration and assay incubation time, preliminary experiments were performed to determine the sensitivity of the stably transfected cell line to functional ricin exposure relative to a similar CHO cell line. In the initial experiments, pure ricin procured from Vector Labs was used to prepare an eight-point 1:5 dilution series (i.e., 2,000 to 0.02 ng/mL). Ricin dilutions were prepared in DMEM media (lacking serum and all additives). However, in subsequent experiments, eight-point 1:2 dilutions were made between 0.02 and 40 ng/mL. A typical assay involved seeding a 100  $\mu$ L aliquot containing  $\sim 10^4$  of washed cells and 25  $\mu$ L of dilute ricin. The final selected double transfectant cell line (Clone 1–2) and CHO cell lines were seeded in triplicate in a 96-well plate. In the initial set of experiments, ricin was added after 24 h of cell seeding. The cells were lysed and assayed for luciferase activity after 24 h of seeding. In subsequent experiments, ricin was added at time zero (the time of cell seeding).

## 2.7 Primary Data Acquisition and Analysis

Luminescence intensity was measured on a Promega GloMax luminometer and expressed as Relative Light Units (RLU). Output data from the luminometer was exported in Excel file format. Reduction of data included subtraction of background (as determined from uninduced control cells) from each data point. Duplicate and triplicate data points were averaged, and inhibition was calculated as a percentage of luciferase expression of average signal from induced cells.



## 2.8 Coupon Inoculation and Sample Preparation

Baseline experiments were performed to determine ricin recovery efficiency from coupons and appropriate dilution for neutralized disinfectant samples. Recovery efficiency was compared using three extraction solutions, buffered peptone water (BPW), water, and water containing 0.05% Tween 80. No significant difference in toxin recovery was observed; therefore, water was used as the extraction solution. To determine recovery efficiency, coupons were spotted with 25  $\mu$ L of pure or crude ricin (see Section 3.2.3) and allowed to dry overnight in a bio-safety level 2 cabinet. Inoculated coupons were dropped in 10 mL extraction solution and vortexed for 2 min. Serial dilutions of extracted samples were made in DMEM, then 25  $\mu$ L of diluted samples were added to wells in a 96-well plate containing  $10^4$  seeded cells per well. The appropriate dilution necessary to avoid cell toxicity from neutralized disinfectant samples was also determined. This was done by dropping ricin inoculated coupons in 10 mL of neutralized disinfectant (see Section 2.9) and vortexing each sample for 2 min. One undiluted and one 1:50 diluted aliquot from each sample were evaluated as described for recovery experiments. The appropriate dilution was chosen based on the dilution level at which no toxic effects of neutralized decontaminant were observed as measured by inhibition of luciferase expression.

## 2.9 Decontamination Assay

The optimized bioassay was used to determine the efficacy of decontamination of coupons inoculated with ricin. Three commercial disinfectants were tested for their ricin decontamination efficacy: 1:20 diluted bleach, 1% hydrogen peroxide, and 250 ppm chlorine dioxide. Steel coupons (1  $\text{cm}^2$ ) were spotted with 25  $\mu$ L crude ricin containing 35 mg/mL total protein (equivalent to 0.2 mg pure ricin) and allowed to dry overnight in a bio-safety level 2 cabinet. Inoculated coupons were then dropped in 7.5 mL of 1% hydrogen peroxide, 1:20 diluted bleach, or 250 ppm chlorine dioxide. After 30 s, 2.5 mL of 2 M sodium thiosulfate was added as a neutralizer. A control for each disinfectant was conducted by adding 25  $\mu$ L of crude ricin extract to 10 mL of neutralized disinfectant. Neutralized samples were vortexed for 2 min to extract ricin from the steel coupon. The extracted ricin samples were serially diluted in cell media, and 25  $\mu$ L of diluted samples were added to a 96-well plate containing  $10^4$  cells (in 100  $\mu$ L volume) per well. Plates were incubated for 24 h at 37  $^{\circ}$ C and then developed by adding 125  $\mu$ L of luciferase reagent. The contents of each well were mixed three times using a multichannel pipettor. Plates were assayed for luminescence immediately after mixing.

## 2.10 Ricin Limit of Detection Estimations

The ricin standard samples were made using a pure ricin stock from Vector Labs. The standard dilution series was prepared just prior to each assay, and samples ranged from 0 to 80 ng/mL. An aliquot of 100  $\mu$ L of cells and 25  $\mu$ L ricin sample were added and mixed per well. Therefore, a total of 125  $\mu$ L volume in each well contained between 0–2 ng ricin.

### 3. RESULTS

#### 3.1 Cell Line Clone Selection

Initially, a total of 33 clones were selected on the basis of their growth in the presence of hygromycin. However, eight of these clones did not grow well, and were not carried further. The remaining 25 clones were screened for induction of luciferase expression in the absence of doxycycline. In addition, each clone was assessed for any background expression present in the uninduced state. As a positive control, a CHO cell line stably transfected with the luciferase gene under control of the tetracycline-responsive element was also included. The results are summarized in Table 2. As seen in the table, many of the clones show only 1- to 2-fold induction of luciferase activity. The highest level of expression, with the lowest background, was seen with Clone 1-2, which showed 1,000-fold induction. Clone 1-2 was selected based on growth rate, high expression of induced luciferase, and low background in the uninduced state. This clone was used for optimization of the ricin bioassay.

**Table 2. Clonal Selection Results**

Clone #	UNINDUCED		INDUCED		Fold Induction
	Average RLU	SD	Average RLU	SD	
1-1	32	5	56	4	2
1-2	42	5	43434	10239	1040
1-3	36	6	38	11	1
1-4	35	6	37	3	1
1-5	26	3	28	6	1
1-6	29	7	75	102	3
1-7	17	4	30	9	2
2-1	36	5	92	10	3
2-2	31	3	525	464	17
2-3	42	6	33	5	1
2-5	25	4	23	3	1
2-6	32	3	16	5	0
2-7	44	44	23	5	1
2-8	93	132	24	7	0
2-9	23	2	21	5	1
3-1	95	21	6644	492	70
3-3	28	6	168	52	6
3-4	31	5	53	10	2
3-5	210	41	16149	899	77
4-1	34	9	337	47	10
4-2	28	8	6182	1227	221
4-3	30	3	113	19	4
4-5	29	6	45	31	2
4-6	39	18	35	8	1
4-7	29	8	42	18	1

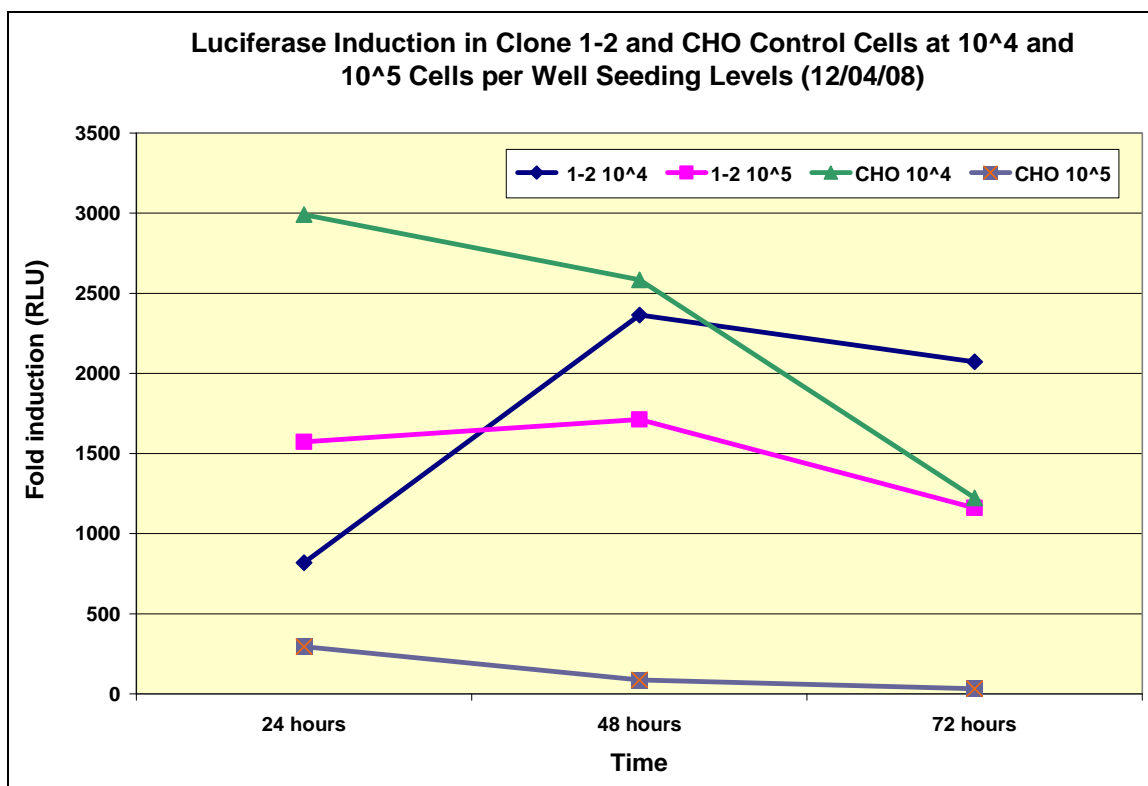
## 3.2 Assay Optimization

Several parameters were evaluated to optimize the ricin bioassay. These parameters included general issues associated with the assay setup such as cell concentration, incubation period, time of addition for ricin, ricin standard curve concentration and preparation, and processing of decontamination samples. Initial optimization experiments also included a comparison with a CHO control cell line.

### 3.2.1 Cell Concentration and Incubation Time

To determine the optimal cell number for the assay and the optimal time to incubate between cell seeding and assay development, cells were seeded at two concentrations,  $10^4$  or  $10^5$  cells per well, and the cells were allowed to grow for 24, 48, or 72 h before the plates were developed. Clone 1-2 and the CHO control cell line were included in this series of experiments. The results are summarized in Figure 2.

As shown in Figure 2, the highest levels of luciferase induction for Clone 1-2 were seen for  $10^4$  cells at 48 and 72 h. However, sufficient levels of induction were seen as early as 24 h. The fold induction levels at 24, 48, and 72 h for  $10^4$  cells ranged from 800 to 2,300 RLU. Between 0 and 48 h, the Clone 1-2 cells, seeded at an initial concentration of  $10^4$  cells per well, showed a marked increase in luciferase production, suggesting that this cell concentration results in healthy cell activity. Hence, this cell concentration was the highest optimal cell density for this assay. Although the levels for luciferase induction were higher for  $10^5$  cells at 24 h, the general trend in this time-course experiment at this cell density showed a decrease in luciferase expression over time. This suggests that seeding at the higher density results in overcrowding of cells, which affects cell viability and transcription activity. Based on these results, a 24 h incubation and cell count of  $10^4$  cells/well for Clone 1-2 were determined to be optimal and were, therefore, selected as experimental conditions for subsequent work.

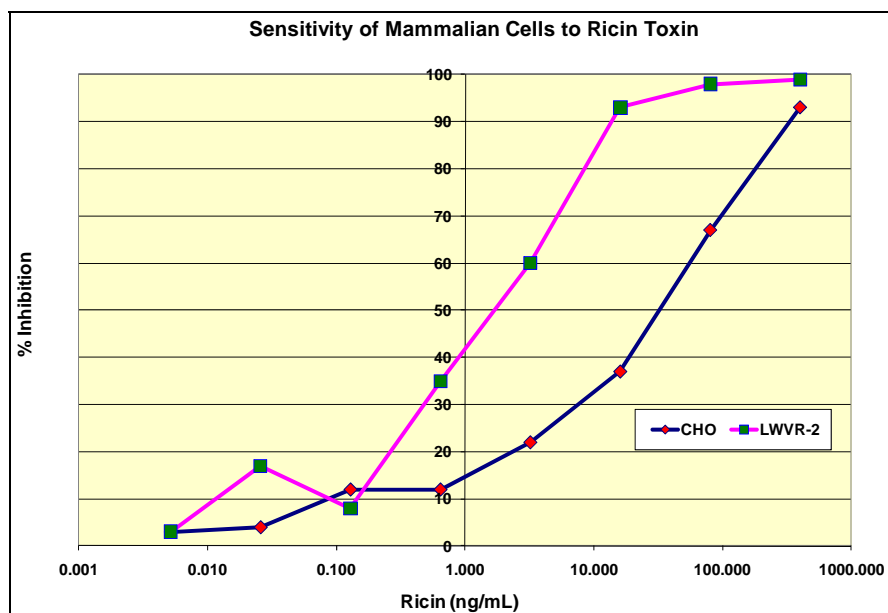


**Figure 2. Effect of Cell Number and Incubation Period on Luciferase Expression**

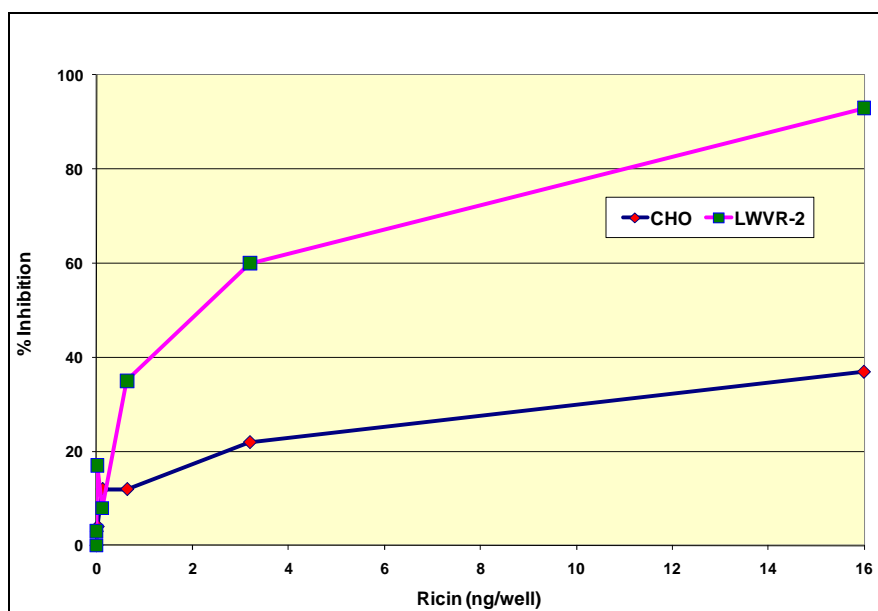
### 3.2.2 Sensitivity of Cell Line to Pure Ricin Standards

Sensitivity of both cell types (Clone 1–2 and CHO) to various concentrations of ricin is summarized graphically in Figures 3 and 4. As seen in Figure 3, the recombinant 1–2 cell line is significantly more sensitive to functional ricin than the control CHO cell line. In addition, the 1–2 cell line demonstrated a 50% inhibition in luciferase activity in the presence of ~3 ng/mL. The limit of holoricin detection by Clone 1–2 is 0.6–0.8 ng/mL. In comparison, the CHO cell line showed a similar level of inhibition in the presence of 40–50 ng/mL. The same data are re-plotted to indicate the inhibitory response in the presence of 0–16 ng/mL of holoricin (Figure 4).

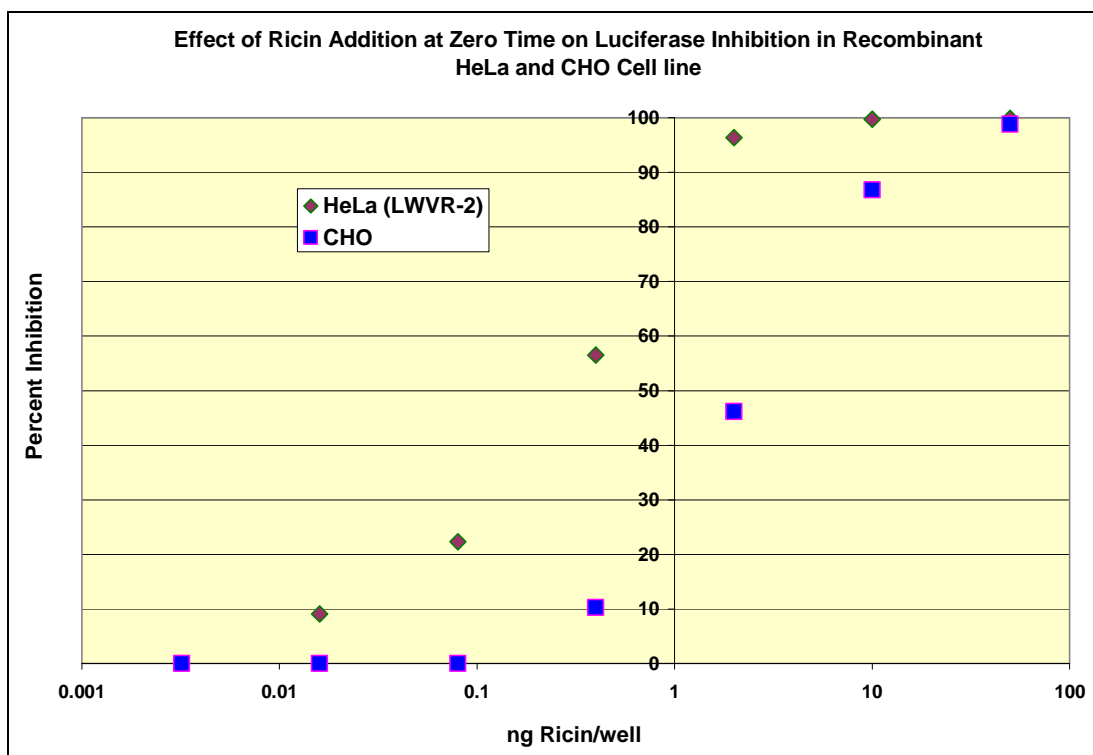
Optimization of the bioassay included determining the optimal time, following cell seeding, to add ricin samples. Initially, ricin samples were added 24 h after the cells were seeded (Figures 3 and 4). However, as seen in Figure 5, it was shown that the addition of ricin at the time of cell seeding has no effect on assay sensitivity.



**Figure 3. Sensitivity of Two Cell Lines to varying amounts of holoricin**

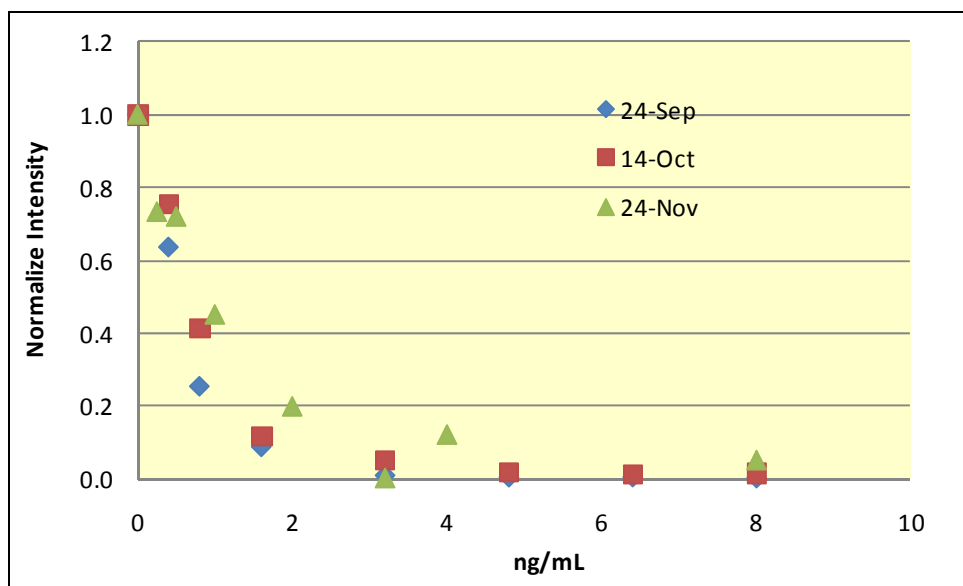


**Figure 4. Effect of low levels of holoricin on Luciferase Expression**

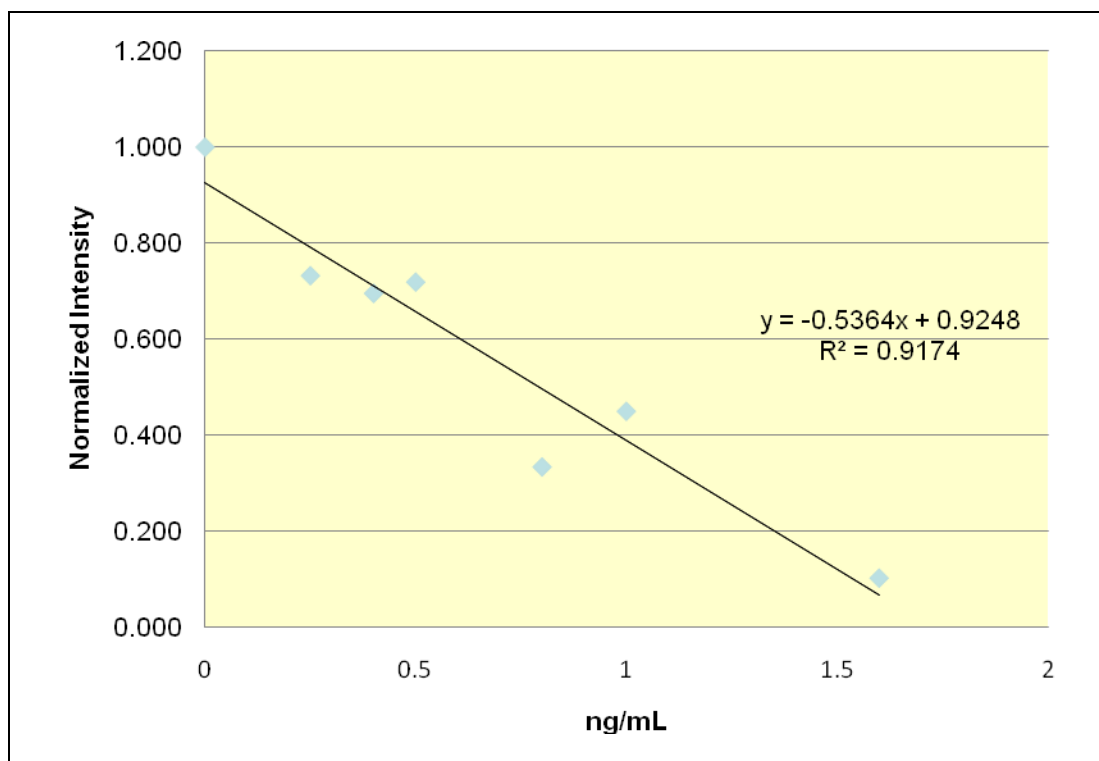


**Figure 5. Optimization of ricin addition**

The reproducibility of ricin cytotoxicity to recombinant luciferase HeLa cells was evaluated over a two month period with three independent experiments. The change in luciferase expression in HeLa cells after 24 h incubation with ricin concentrations from 0 to 8 ng/mL is shown in Figure 6. The relative luminescence at 0 ng/mL ricin concentration was used to normalize the luminescence values in Figure 6. The average standard deviation between the three experiments for each data point was 0.04. The cellular luciferase activity in Clone 1–2 cells significantly decreased at ricin concentrations >1.6 ng/mL. The linear response of HeLa cells to ricin cytotoxicity is shown in Figure 7, which illustrates that luciferase expression is almost completely inhibited in the presence of >8 ng/mL of ricin. The correlation coefficient of the linear portion of the cytotoxicity curve is 0.91, indicating an adequately linear relationship between ricin concentration and normalized cellular luminescence. Cellular luciferase activity becomes significantly nonlinear at ricin concentrations >8 ng/mL.



**Figure 6. Response Reproducibility of Clone 1-2 Cells to Ricin**



**Figure 7. Linear Relationship between Clone 1-2 and Ricin Concentration**

### 3.2.3 Decontamination Sample Preparation

For the decontamination assays, crude ricin was obtained from ECBC's BioPhysical Chemistry Group. Based on the protein estimation, the crude ricin sample was 0.5% ricin (0.2 mg ricin and 35 mg protein/mL). Based on luciferase activity measurements of the crude ricin extract (data not shown) and the linear ricin response curve generated with known quantities of ricin (Figure 7), it was estimated that the crude ricin stock contained ~0.56% active ricin.

Prior to analyzing disinfected coupons inoculated with crude ricin in the optimized bioassay, experiments were performed to determine if neutralized disinfectants had any toxic affect on the cells and if samples could be diluted to abate this effect. Table 3 shows that all three neutralized disinfectants at zero dilution resulted in full inhibition of luciferase activity. A likely explanation for this outcome is the high salt concentration that may result in hypertonic conditions for the cells, which leads to loss of viability after 24 h incubation in the neutralized disinfectant solution. A 1:50 dilution of the neutralized disinfectants in cell media alleviated the osmotic effects of the salts on the cells and the cells remained viable after 24 h incubation.

**Table 3. Effect of Neutralized Disinfectant on Luciferase Induction**

<b>Dilution</b>	<b>Control</b>	<b>Chlorine Dioxide 250 ppm</b>	<b>Bleach 1:20 dil.</b>	<b>Hydrogen Peroxide 1%</b>
0	0	100	100	100
1:50	0	0	0	0

Percent inhibition of luciferase activity at zero and 1:50 dilution in cell media

### 3.3 Ricin Decontamination with Decontamination Solutions

Following thorough optimization of the bioassay, three disinfectant solutions were tested for their ability to decontaminate coupons inoculated with crude ricin samples. The results of the disinfection experiments are shown in Table 4. For neutralized disinfectant controls, crude ricin extract had >92% luciferase inhibition. The neutralized solution did not adversely affect ricin toxicity to HeLa cells. Greater than 80% active ricin was recovered from the positive control coupons that were not exposed to disinfectant. The results indicated that 30 s contact with a 1:20 diluted bleach or 250 ppm chlorine dioxide caused complete inactivation of ricin toxic effects to HeLa cells. Conversely, a 30 s contact time with 1% hydrogen peroxide had partial effect on the ricin toxicity to the cells. This ricin sample resulted in 62% inhibition of luciferase expression, indicating that active ricin was still present following exposure to 1% hydrogen peroxide.



**Table 4. Decontamination of Crude Ricin on Steel Coupons with Bleach, Chlorine Dioxide, and Hydrogen Peroxide**

<b>Test Decon Solution</b>	<b>Ricin Recovery (%)</b>	<b>Estimated Ricin Remaining (ng)</b>	<b>Comments</b>
1:20 Diluted bleach	80	0	No pH adjustment
250 ppm CD Solution	97	0	No ricin detected (LOD = <0.2 ng)
1% H <sub>2</sub> O <sub>2</sub>	94	1900	Partial Decon

Active ricin remaining after 30 s contact time with disinfectants. Each coupon was contaminated with 35 mg total protein containing 5 µg active ricin and exposed to 30 s of contact time with each of the listed disinfectants.

#### 4. DISCUSSION AND CONCLUSIONS

The purpose of this project was to develop a sensitive and activity-based cell assay for the detection of functional ricin following decontamination of building interior surfaces. The strategy for developing the cell assay included the stable transfection of a reporter gene, luciferase, into an engineered HeLa cell line. The stable transfection of the cell line and the subsequent optimization of the bioassay have successfully been achieved. The basis for the newly developed assay was inhibition in the expression luciferase in the presence of functional ricin. Although only ricin was tested in this project, this assay could potentially be used to detect the presence of any toxin or chemical affecting gene expression.

A total of 33 stably transfected clones were screened for inducible expression of luciferase with low basal expression. Clone 1–2 was selected for further optimization based on these criteria. This clone was used for optimization experiments to determine optimal cell seeding concentration, assay incubation time, time of ricin addition, and decon sample procedures. In addition, the engineered cell line was determined to be highly stable, as assessed by no change in expression level over a 6 month period.

The bioassay is highly sensitive because it can detect active ricin at concentrations as low as 0.6–0.8 ng/mL. The assay is easy to set up and requires the following steps:

- a. 2 h for preparation of cells inducing luciferase (by removal of doxycycline) and toxin addition and 18–24 h incubation
- b. 15 min for cell lysis and light output readout

For high-throughput analysis, the assay can be easily automated while maintaining high precision by using a robotics system to process multiple plates. The inhibition in luciferase expression is closely related to the presence of low amounts (<1 ng/mL) of holoricin.

Following optimization, the efficacy of three disinfectants in ricin decontamination was determined. A number of parameters had to be optimized before a decon protocol was developed, such as complete neutralization of the active moiety within the decon solution and cell number for the assay. The data show that two of the three disinfectants successfully decontaminated ricin-contaminated steel coupons within 30 s. Based on the results, we conclude that this assay can be used for the detection of low residual active ricin remaining on surfaces following decontamination. It will also be of interest to extend this assay to other building interior surfaces.

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## APPENDIX

### General Ricin BioAssay Protocol

